Leukemia inhibitory factor inhibits neuronal terminal differentiation through STAT3 activation

Cheil Moon*†, Joo-Yeon Yoo†‡§, Valéry Matarazzo*, Young Kwan Sung*, Esther J. Kim*, and Gabriele V. Ronnett*¶‖

Departments of *Neuroscience and ¶Neurology, ‡Howard Hughes Medical Institute, and §Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

Edited by Solomon H. Snyder, The Johns Hopkins University School of Medicine, Baltimore, MD, and approved May 9, 2002 (received for review March 6, 2002)

The discovery of stem cells in the adult central nervous system raises questions concerning the neurotrophic factors that regulate postnatal neuronal development. Olfactory receptor neurons (ORNs) are a useful model, because they are capable of robust neurogenesis throughout adulthood. We have investigated the role of leukemia inhibitory factor (LIF) in postnatal neuronal development by using ORNs as a model. LIF is a multifunctional cytokine implicated in various aspects of neuronal development, including phenotype determination, survival, and in response to nerve injury. LIF-deficient mice display significant increases, both in the absolute amount and in the number of cells expressing olfactory marker protein, a marker of mature ORNs. The maturation of ORNs was significantly inhibited by LIF *in vitro*. LIF activated the STAT3 pathway in ORNs, and transfection of ORNs with a dominant negative form of STAT3 abolished the effect of LIF. These findings demonstrate that LIF negatively regulates ORN maturation via the STAT3 pathway. Thus, LIF plays a critical role in controlling the transition of ORNs to maturity. Consequently, a population of ORNs is maintained in an immature state to facilitate the rapid repopulation of the olfactory epithelium with mature neurons during normal cell turnover or after injury.

The concept that neurons are terminally differentiated and not replaced in the adult central nervous system was challenged with the discoveries of stem cells and neurogenesis in the adult mammalian nervous system (1–5). This finding raises the possibility that stem cells may be manipulated in the context of factors that regulate neurogenesis during adulthood to replace damaged neurons. However, our understanding of these factors is limited.

Several classes of factors are implicated in neurogenesis. The most extensively studied factors involved in neuronal development are neurotrophins (6). Neurotrophins act by means of receptor tyrosine kinases and regulate neural survival, function, and plasticity. Neuropoietic cytokines are also implicated in neurogenesis (7). Different from neurotrophins, cytokines act by means of cytosolic tyrosine kinases. Members of this family interact with multimeric cell surface receptors containing as a common glycoprotein subunit termed gp130 (8). A member of this family, leukemia inhibitory factor (LIF), is a multifunctional cytokine originally identified as a factor that inhibits proliferation and induces differentiation of macrophages (9). LIF acts on neurons as well as many other cell types. LIF influences neuronal survival (10) and differentiation in the peripheral nervous system (11), induces the switch of neurotransmitter expression in sympathetic neurons (12–14), stimulates glial development (15, 16), is induced in sensory neurons in response to trauma (17–19), and is up-regulated in olfactory epithelium after bulbectomy (20, 21).

To evaluate factors that regulate neuronal development, a number of models have been developed. Because of its cytoarchitecture, ongoing postnatal neurogenesis, and the availability of *in vitro* models, the olfactory epithelium is a useful model to evaluate factors that regulate in particular postnatal neuronal development (22). The olfactory epithelium is composed of immature and mature olfactory receptor neurons (ORNs), nonneuronal sustentacular cells, and basal cells, some of which are progenitor cells for ORNs (23–27). Although a number of factors are implicated as relevant to various aspects of olfactory neurogenesis, the factors that control the transition from immature to mature neuron are unknown. This is an important transition point, as the maintenance of a population of immature neurons (as opposed to basal or stem cells) facilitates the rapid repopulation of the olfactory epithelium with mature neurons during normal cell turnover or after injury.

As they differentiate, ORNs move apically in the epithelium, permitting determination of neuronal age by position. As they mature, ORNs express olfactory transduction mechanisms, such as adenylyl cyclase type III (ACIII) (28, 29), an olfactory receptor neuronal-enriched G protein (G_{olf}) (30), and olfactory marker protein (OMP). OMP is a cytosolic acidic protein found in ORNs (31, 32). OMP is present only in differentiated ORNs (31), and thus OMP is widely accepted as a marker for maturity. Here, we investigated the influence of LIF on postnatal neuronal development by using ORNs as a model system.

Materials and Methods

LIF-Deficient Mice. LIF-deficient mice are a kind gift from S. Landis at the National Institute of Neurological Disorders and Stroke (Bethesda). Male mice, 4–6 weeks old, were used. All experimental protocols were approved by the Johns Hopkins University Institutional Animal Care and Use Committee, and all applicable guidelines from the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* were followed.

Antibodies. Rabbit anti-ACIII, anti-GFP (green fluorescent protein), anti-gp130, anti-Jak2, anti-LIFRβ, anti-STAT1, anti-STAT3, and anti-Tyk2 (tyrosine kinase 2) antibodies were purchased from Santa Cruz Biotechnology. Anti-phosphoSTAT1 and phosphoSTAT3 (Tyr-705) were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal mouse antibody against monomeric actin was obtained from Developmental Studies (Hybridoma Bank). Goat anti-OMP and rabbit anti-O/E-1 (Olf-1/EBF-like protein) antibodies were kind gifts from F. Margolis (University of Maryland, Baltimore) and R. Reed (Johns Hopkins University), respectively.

Primary Culture of ORNs. Cultures were prepared as described (33), with some modifications. Cells were plated at a density of 6×10^5 cells per cm^2 on culture plates coated with 25 μg/ml laminin

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: LIF, leukemia inhibitory factor; CNS, central nervous system; ORN, olfactory receptor neuron; OMP, olfactory marker protein; Jak, Janus family tyrosine kinase; STAT, signal transducers and activators of transcription; O/E-1, Olf-1/EBF-like protein; SIE, sis-inducing element; LIFRβ, LIF receptor β; gp130, glycoprotein 130; OSM, oncostatin M; CTNF, ciliary neurotrophic factor; GFP, green fluorescent protein.

*C.M. and J.-Y.Y. contributed equally to this work.

‖To whom reprint requests should be addressed. E-mail: gronnett@jhmi.edu.

NEUROBIOLOGY

(Collaborative Research). Cultures were placed in a humidified 37°C incubator receiving 5% CO_2. On day 2 and every day thereafter, cells were fed with modified Eagle's medium containing D-valine (MDV, GIBCO/BRL), 0.5% dFCS, 50 units/ml gentamycin, 100 μg/ml kanamycin, and 2.5 μg/ml amphotericin B. Depending on culture conditions, nerve growth factor (NGF) or LIF were added to the culture medium at concentrations of 25 ng/ml and 10 ng/ml, respectively.

***In Situ* Hybridization.** To generate cRNA probes, plasmid (pAC-CMV) containing the full coding sequence for mouse LIF cDNA was used. The plasmid was a kind gift from P. Patterson (California Institute of Technology, Pasadena). The plasmid was subcloned into pBluescript SK (Stratagene). For OMP, the entire coding sequence of OMP cloned in the Bluescript plasmid (kind gift from F. Margolis) was used. Sense and antisense digoxigenin-labeled cRNA probes were generated by *in vitro* transcription with T3 and T7 RNA polymerase (Boehringer Mannheim). Because of its long size, LIF transcripts were base hydrolyzed to an average size of 700 bp and used for the study. *In situ* hybridization was performed according to published protocols (34) with modifications. Immunoreactivities were detected by alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim; 1:2,500 dilution), nitroblue tetrazolium, and X-phosphatase color detection (Boehringer Mannheim). To distinguish olfactory epithelium from regions of the developing respiratory epithelium, adjacent sections of developing tissue were analyzed by immunohistochemical localization of OMP.

Gel Electrophoresis and Immunoblot. Protein samples prepared from nasal turbinates of adult rat, mouse, or total lysates of primary cultures were subjected to SDS/PAGE (protein loaded: rat 40 μg, mouse 30 μg, primary culture 5×10^3 cells), transferred to nitrocellulose membrane, and probed with primary antibodies. The primary antibodies were used at 1:1,000 dilutions, except anti-OMP antibody (1:2,500). The secondary antibodies were horseradish peroxidase-conjugated IgGs (Boehringer Mannheim; 1:10,000 dilution). The immunoblots were visualized by using enhanced chemiluminescence kit (Amersham Pharmacia). For quantification analysis, film was scanned on UMAX Powerlook 1100 as tiff format and quantified by using NIH IMAGE software (version 1.62; http://rsb.info.nih.gov/nih-image).

Gel Retardation and Supershift Assay. The double-stranded DNA probe for STAT3 binding was obtained from Santa Cruz Biotechnology. The probe sequence is designed from the consensus DNA sequence of SIE (sis-inducing element), 5′-GATCC**TTCT-GGGAA**TCCTAGATC-3′ with the DNA binding sites indicated in boldface. After stimulation of ORN primary cultures with cytokines, nuclear extracts were isolated as described (35), mixed with radiolabeled probe, and incubated for 15 min. Samples were run on nondenaturing 4% polyacrylamide gels, dried, and exposed to X-Omat films (Kodak). For antibody supershift assays, nuclear extracts were mixed with anti-STAT3 antibodies at 1:25 dilution.

Immunohistochemistry. Mice are anesthetized with xylaket (45 mg/kg, i.p.) and perfused transcardially with PBS followed by Bouin's fixative (Sigma). Olfactory tissues are dissected and postfixed for 2 h in Bouin's fixative and then sequentially sunk in 10%, 20%, and 30% (wt/vol) sucrose each for 24 h. Blocks are embedded in Tissue-Tek (Miles, Elkhart, IN), stored at −70°C until use, and then sectioned in a cryostat at 18 μm. For primary cultures on slides, ORNs were fixed with an ice-cold mixture of methanol/acetone (50:50). Labeling with primary antibodies was detected by using an avidin-biotin-peroxidase kit (Elite kit, Vectastain) and visualized with diaminobenzidine or other chromagens. The primary antibodies were used at 1:1,000 (O/E-1), 1:5,000 (OMP), and 1:200 dilutions (phospho-STAT3).

Immunofluorescence. Primary cultures of ORNs on glass coverslips were fixed with an ice-cold 4% paraformaldehyde in 4% sucrose for 20 min at 4°C. Permeabilization was followed in 0.2% Triton X-100 for 10 min at 4°C. Then, cells were incubated in 100% methanol for 10 min at 4°C. After rinsing three times with PBS, cells were blocked with 10% normal donkey serum for 60 min at room temperature, and incubated with rabbit anti-GFP (1:2,000) and goat anti-OMP (1:5,000) antibodies overnight at 4°C. On the next day, cells were washed with PBS three times, and incubated with donkey anti-goat rhodamine-conjugated antibody (1:100) and anti-rabbit fluorescein-conjugated antibodies (1:50) (Jackson ImmunoResearch) in 10% normal donkey serum for 1 h at room temperature. The slides were rinsed with PBS three times, and mounted with aquapolymount.

Construction and Transfection of pEGFP-STAT3(Y705F) Vectors. Dominant negative STAT3 was obtained by mutation of Stat3 Tyr-705 to phenylalanine (Y705F) (36). To construct pEGFP-STAT3 (Y705F) vector, the coding sequence of mouse STAT3 (Y705F) was fused in frame with the pEGFP-C1 plasmid (CLONTECH). Transfections of vectors were performed by using Helios Gene Gun System (Bio-Rad). DNA vectors were coated on gold microcarriers (0.6 μm). For the transfection experiments, primary cultures of rat ORNs were prepared and incubated in 15% dFBS medium without neurotrophic factors overnight. On the next day, the medium was changed to a medium containing 0.5% dFCS medium. Then, the DNA/microcarriers were fired at the cultures. The cells were incubated in the conditioned medium for 16 h, and then changed into media containing NGF (25 ng/ml) either with or without LIF (100 ng/ml).

Results

OMP Expression Is Increased in LIF-Null Mice. Understanding the functions of LIF has been facilitated by the availability of mice with targeted disruption of the LIF gene (37). We first examined the expression of OMP in the olfactory epithelium of LIF-deficient mice (Fig. 1). Consistent with previous reports (31, 38), OMP was expressed in the most apical ORNs, and decreased in ORNs located in more basal regions of the olfactory epithelium of wild-type (WT) mice (Fig. 1*a*). In LIF-deficient mice, OMP was increased, in terms of the intensity of immunoreactivity (Fig. 1*b*) and in terms of the absolute amount as determined by Western blot analysis (Fig. 1*g*). In addition, the number of OMP-positive cells was significantly increased in the olfactory epithelium compared with WT littermates (169.90% ± 4.83%, $P < 0.0\,01$; Fig. 1*h*). We next examined the expression of ACIII, a protein expressed in the cilia of mature ORNs in olfactory epithelium (29). As ORNs mature, the cilia projects into the nasal lumen (28, 39). ACIII converts environmental signals (odors) into a biochemical signal (cAMP) (28). ACIII was highly localized to neuronal cilial layer in WT mice, as reported (28, 29) (Fig. 1 *c* and *d*). Compared with WT mice, LIF-deficient mice displayed higher intensity of ACIII immunoreactivity (Fig. 1*d*), and about 60% increased expression of ACIII as determined by Western blot (Fig. 1*g*).

An increase in the number of OMP-positive cells in LIF null mice could reflect one of several disturbances to olfactory receptor neuronal development, including a global increase in the number of cells of the ORN lineage, or an increase specifically in the number of mature ORNs in the population. O/E-1 is a transcription factor expressed in cells of the ORN lineage, and plays an important role in ORN differentiation (40). O/E-1 is therefore a useful marker to identify the total number of cells in the ORN lineage present in the epithelium. O/E-1 immuno-

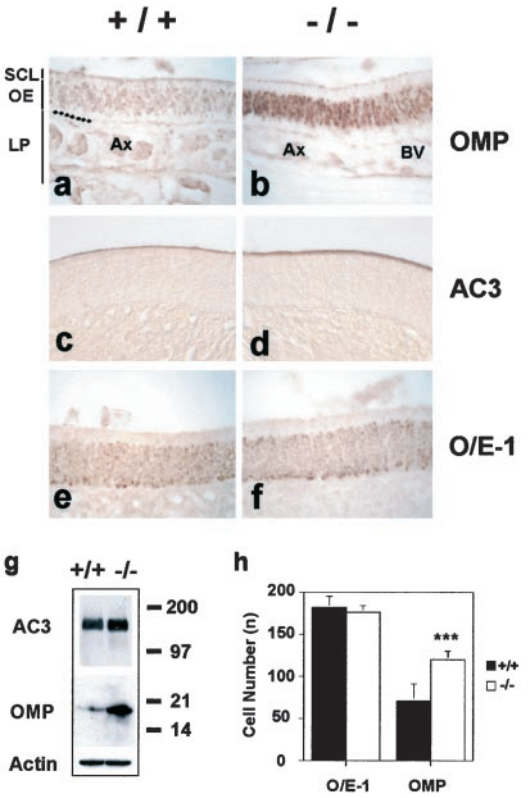

Fig. 1. Expression of OMP, ACIII, and O/E-1 in olfactory epithelium. (*a–d*) Immunoreactivity was assessed in WT (*a*, *c*, and *e*) and LIF-deficient mice (*b*, *d*, and *f*) for OMP (*a* and *b*), ACIII (*c* and *d*), and O/E-1 (*e* and *f*). Ax, axon bundle; BV, blood vessel; OE, olfactory epithelium; LP, lamina propria; SCL, sustentacular cell layer. The dotted line represents the basal lamina. (*g*) Expression of OMP and ACIII in WT (+/+) and LIF-null mice (−/−) was assessed by immunoblot. Equal loading of the protein samples was confirmed by probing for monomeric actin. (*h*) Summary of OMP and O/E-1 staining in olfactory epithelium. Cells in areas of 0.04 mm^2 (0.2 × 0.2 mm; width × height) were counted. Data represent the mean ± SEM of cell numbers counted in at least 10 different areas from three independent experiments. Statistical significance is indicated as *** ($P < 0.0001$, Student's *t* test).

staining was similar in the olfactory epithelium of WT and LIF-null mice (Fig. 1 *e* and *f*). The number of cells expressing O/E-1 was not significantly different between WT and LIF null mice (Fig. 1*h*). These results suggest that LIF does not influence the absolute number of ORNs, but affects the expression of OMP and ACIII, and hence the degree of maturation of ORNs.

LIF and Components of Its Signaling Cascade Are Expressed in ORNs. To gain insight into the mechanism by which LIF influences ORN maturation, the expression of LIF and its signaling pathways in the olfactory epithelium was determined (Fig. 2). LIF mRNA was present in the mouse olfactory epithelium (Fig. 2*a*). LIF message was expressed throughout most layers of the epithelium where immature and mature ORNs are expressed, excluding regions occupied by sustentacular cells or basal cells (Fig. 2*a*). No signal for LIF message was detected by using olfactory epithelium from LIF-null mice (Fig. 2*b*), or when sense probes for LIF on olfactory epithelium from WT or LIF-null mice were used (Fig. 2 *c* and *d*, respectively). OMP was used as a control to visualize the distribution of mature neurons (Fig. 2 *e* and *f*); expression of OMP is absent from immature neurons and neuronal precursors located in the lower one-third of the epithelium.

LIF binds to a receptor complex consisting of LIF receptor β

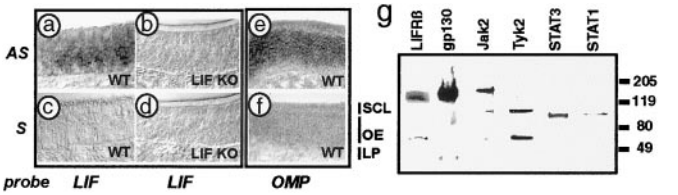

Fig. 2. LIF expression in olfactory epithelium. (*a–f*) *In situ* hybridization analysis of LIF in olfactory epithelium. Expression of LIF mRNA was determined in coronal sections of adult mouse nasal cavity by using antisense LIF riboprobe (*a* and *b*). To ascertain the specificity of the probe, sense controls were also performed (*c* and *d*). Expression of OMP in olfactory epithelium was also examined by using antisense and sense OMP riboprobe (*e* and *f*, respectively). (*g*) Immunoblot analyses of LIF signaling components in olfactory tissues. The molecular mass (in kDa) is indicated at right.

(LIFRβ) and glycoprotein 130 (gp130) to activate Jak (Janus family tyrosine kinase), which in turn phosphorylates STATs (signal transducers and activators of transcription) (41, 42). LIFRβ, gp130, Jak2, tyrosine kinase 2 (Tyk2), STAT1, STAT3 were all expressed in the rat olfactory tissues (Fig. 2*g*). As *in vitro* culture models were used in this study, total lysates from primary cultures of ORNs were also analyzed. The expression of LIFRβ, gp130, Jak2, Tyk2, STAT1, and STAT3 was also detected in primary cultures of ORNs (data not shown). Thus, LIF and its signaling components are positioned to mediate autocrine/paracrine effects on ORN maturation. We also observed expression of LIFRα, Jak1, and STAT5b in ORNs by reverse transcription–PCR analysis, even though we could not detect them by Western blot (data not shown). Expression of other STAT family members intimates that other cytokines may also affect the olfactory system by means of the activation of STAT1 and/or STAT5b.

LIF Activates the Jak/STAT Pathway in ORNs. We wished to determine the signaling pathway that might mediate the inhibitory effects of LIF on ORN maturation. We used dissociated primary cultures containing ORNs (33). We used phospho-specific STAT3 antibodies, and demonstrated that LIF (10 ng/ml) induced STAT3 phosphorylation on tyrosine 705 (Fig. 3*a*). The LIF-induced STAT3 activation was blocked by preincubation with K252a (100 nM), a cell-permeable tyrosine kinase inhibitor. The effect of LIF was dose-dependent (data not shown). Even though STAT1 was expressed in ORNs, LIF did not activate STAT1 (data not shown). On stimulation, STATs are phosphorylated, dimerize, translocate into the nucleus, bind to DNA, and regulate gene expression (43). In primary ORN cultures, only the cells treated with LIF showed an increase in tyrosyl phosphorylated STAT3 (Fig. 3*b*) and a translocation of activated STAT3 into the nucleus (Fig. 3*c*). Electrophoretic mobility shift assay showed that a low-level of constitutive DNA binding (SIE) was present in cells incubated with vehicle alone (Fig. 3*d*). After LIF treatment, STAT3 DNA binding activity was greatly increased. Preincubation with K252a blocked LIF-induced STAT3 binding to DNA. Incubation of complexes with anti-STAT3 antibody resulted in a super shift of the DNA-STAT3 binding complex (Fig. 3*d*). Among other cytokines that share the gp130 receptor, only oncostatin M (OSM) showed significant STAT3 activation, whereas IL-6, ciliary neurotrophic factor (CNTF), and granulocyte colony-stimulating factor were unable to activate STAT3. These results demonstrate that LIF activates STAT3 in ORNs, and that STAT3 is positioned to mediate the inhibitory effects of LIF on the maturation of ORNs.

LIF Inhibits the Expression of OMP *in Vitro*. The overexpression of OMP in the olfactory epithelium of LIF-deficient mice implies

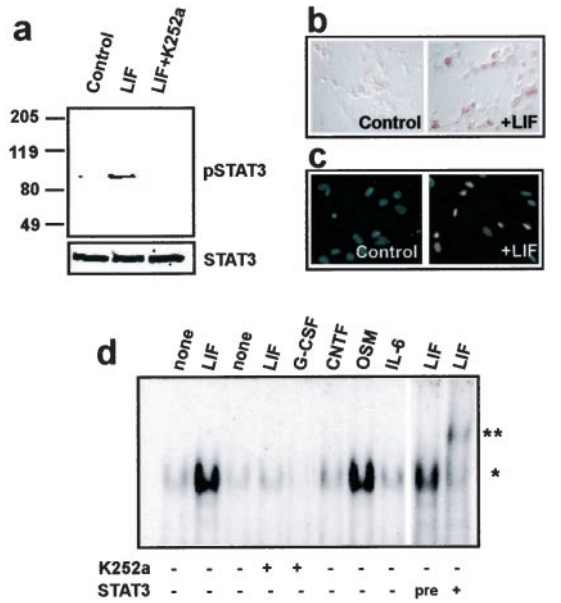

Fig. 3. LIF induced activation of STAT3 in ORNs. (*a*) Western blot analysis of LIF-induced STAT3 activation. Tyrosyl phosphorylated STAT3 was detected by using an anti-phosphoSTAT3 antibody. Primary cultures of ORNs were treated with or without LIF (10 ng/ml) for 15 min. K252a (100 nM) was added before stimulation for 30 min. (*b*) Immunocytochemical analysis of the activation of STAT3 with LIF addition. Primary cultures of ORNs were treated with LIF (10 ng/ml) for 15 min. After fixation, immunocytochemistry was performed using anti-phospho-STAT3 antibodies. (*c*) Immunofluorescence analysis of the translocation of STAT3 into the nucleus on LIF stimulation. Primary cultures of ORNs were treated with LIF (10 ng/ml) for 15 min. After fixation, immunofluorescence was performed with anti-phospho-STAT3 antibodies. The translocation of STAT3 into the nucleus was visualized by using rhodamine-conjugated antibodies and CyQUANT GR dye. STAT3 proteins in the nucleus appear yellow. (*d*) Electrophoretic mobility shift assay. LIF-induced DNA binding activity of STAT3. * indicates SIE-STAT complex. Primary cultures of ORNs were incubated for 15 min as described above. K252a was preincubated for 15 min before stimulation. As a control, no cell lysate was added. The isolated nucleus fractions were incubated with radioactive-labeled SIE and subjected to 4% polyacrylamide, nondenaturing gel electrophoresis. For antibody supershift assay, SIE-STAT3 complex was incubated with anti-STAT3 antibodies (1 μg) for 15 min. ** indicates the supershifted SIE–STAT complex.

that LIF may negatively regulate OMP expression in developing ORNs. To examine this possibility, ORN cultures were incubated in standard medium containing 0.5% serum and either NGF (25 ng/ml) or LIF (10 ng/ml), and analyzed immunocytochemically at later time intervals. NGF is known to be crucial for the survival and development of sympathetic neurons and a subset of sensory neurons (44). In the olfactory system, NGF is reported to be a critical factor for ORN differentiation *in vitro* (33). In control (no growth factors added) or LIF-treated cultures, less than 10% of O/E-1 immuno-positive cells displayed OMP immunoreactivity (Table 1). NGF treatment increased OMP immunoreactivity to approximately 25% of the O/E-1 immuno-positive cells. However, the number of OMP expressing cells was reduced to baseline amounts when LIF was added to NGF-treated cultures. In all culture conditions, the number of O/E-1 expressing cells was not significantly changed, suggesting that the changes in the numbers of OMP-expressing cells did not reflect changes in total cells present because of increased neurogenesis or survival at the times monitored. Thus, *in vitro* data obtained by using ORN cultures confirm *in vivo* data derived by using LIF-null mice, and indicate that LIF negatively regulates OMP expression in ORNs, and hence inhibits the maturation of ORNs.

Dominant Negative STAT3 Inhibits the Effect of LIF in ORNs *in Vitro*. To confirm the role of STAT3 in LIF-mediated inhibition of ORN maturation, we transfected ORNs with a dominant negative form of STAT3 (STAT3 Y705F) (36) fused with GFP. ORNs were transfected with control vector or STAT3 Y705F vector, and incubated in medium containing NGF with (Fig. 4 *d–f* and *j–l*) or without (Fig. 4 *a–c* and *g–i*) LIF. With NGF treatment and vector alone, the number of OMP-positive cells was about 70% of the GFP (+) cells in cultures (67.11% ± 1.76%) (Fig. 4 *a–c* and *m*). When cells were transfected with vector alone, LIF treatment decreased the number of OMP-positive cells to about 50% (36.07% ± 1.33%, $P < 0.0001$) (Fig. 4 *d–f* and *m*). However, LIF treatment was not able to inhibit OMP expression in cells transfected with STAT3 Y705F vector (70.95% ± 5.92%; $P = 0.0004$, when comparing LIF-treated cells transfected with vector to LIF-treated cells transfected with

Table 1. Effect of LIF on ORN maturation

	Treatment			
Cells	None	NGF	LIF	NGF + LIF
OMP (+) cells	9.7 ± 0.5	25.1*** ± 1.1	7.0 ± 0.5	10.5 ± 0.5
O/E-1 (+) cells	97.6 ± 2.3	100.7 ± 6.3	90.8 ± 3.0	100.3 ± 3.6

Olfactory cells were cultured for 4 days in medium containing NGF (25 ng/ml) or LIF (10 ng/ml). Data represents numbers of stained cells. Cells expressing OMP and O/E-1 were determined by using anti-OMP antibodies (OMP) and anti-O/E-1 (O/E-1). Cells were counted in area of 1 mm^2 (1 × 1 mm; width × height). The areas were randomly selected from at least 10 different areas from three independent experiments. Data are mean ± SEM, and statistical significance is indicated as *** ($P < 0.0001$, Student's *t* test).

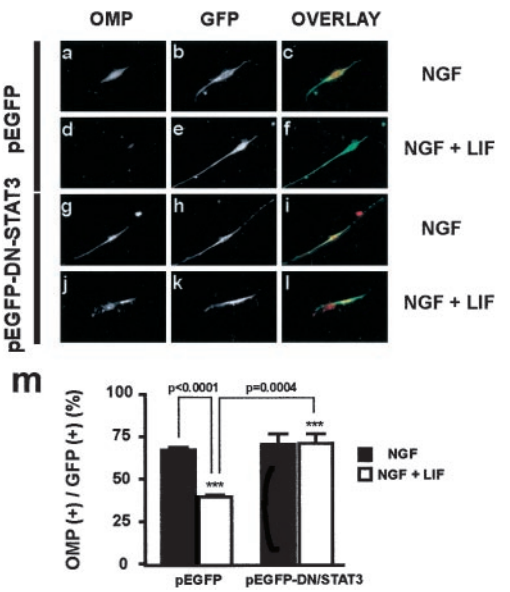

Fig. 4. Transfection of dominant negative STAT3 into ORNs. Transfection of dominant negative STAT3 abolished the effect of LIF on OMP expression of ORNs. a-l. Primary cultures of ORNs were transfected with vectors containing dominant negative STAT3. Transfection of vectors was confirmed by expression of GFP. OMP and GFP were visualized by immunofluorescence. Immunofluorescence was assessed in ORNs transfected with control vector (pEGFP) (*a–f*) and with dominant negative STAT3 vector (pEGFP-D/N STAT3) (*g–l*). ORNs were incubated in medium containing NGF either without (*a–c* and *g–i*) or with LIF (*d–f*, *j–l*). Expression of OMP and GFP was visualized by FITC- and rhodamine-conjugated antibodies respectively, thus, coexpression is visualized as yellow. (*m*) Summary of the effect of dominant negative STAT3 on OMP expression in ORNs. OMP expression in ORNs was significantly decreased in medium containing LIF (100 ng/ml) than in medium containing NGF (25 ng/ml) alone ($P < 0.0001$, Student's *t* test). The effect of LIF was abolished by transfection of dominant negative STAT3 in ORNs ($P = 0.0004$, Student's *t* test). Cell numbers were counted from three independent experiments. Data represent mean ± SEM.

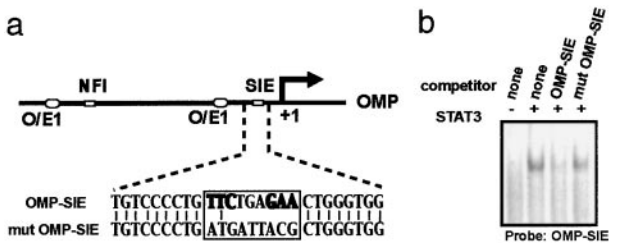

Fig. 5. STAT3 binding to OMP promoter. (*a*) Summary of transcription factor binding sites in OMP promoter region. (*b*) Competitive binding assay. (See text for details.)

STAT3 Y705F vector; Fig. 4 *j–m*). These results indicate that LIF inhibits ORN maturation through the activation of STAT3.

STAT3 Binds to the OMP Promoter Region. The expression of OMP is regulated by tissue-specific transcription factors, including O/E-1 and nuclear factor I (NFI), as is the expression of many of the other known odorant signaling components (45, 46). The promoter region of OMP contains three transcription factor binding sites: two O/E-1 binding sites (distal and proximal sites) and an NFI-like site (Fig. 5*a*). NFI is known to be adjacent to the distal O/E-1 site and negatively regulate gene expression in ORNs (46). By sequence analysis, we identified a potential STAT binding site (SIE) in the promoter regions of OMP (−88 to −79), which is close to proximal O/E-1 sites (Fig. 5*a*). We used purified active STAT3 protein to examine the binding of STAT3 to this region. We generated two competitor probes containing either OMP SIE sequences or mutated OMP SIE sequences. Activated STAT3 bound to the OMP SIE region (Fig. 5*b*). Incubation with cold competitor probes blocked this binding, whereas incubation with a mutated competitor probe could not block binding. This finding suggested that the binding of STAT3 in the OMP SIE region was specific. Thus, STAT3 may binds directly to the OMP promoter region to negatively regulate OMP expression in ORNs.

Discussion

In this study, we demonstrated that LIF-mediated STAT3 activation serves as a negative modulator of ORN differentiation. ORNs senesce throughout life, and are continually replaced from a population of precursor cells. These precursor cells are maintained at various stages of maturation, as identified by developmental markers (22, 47). Thus, mature neurons that are lost through normal turnover or injury can be replaced rapidly by the terminal differentiation of immature neurons already in residence in the olfactory epithelium.

We used both *in vivo* and *in vitro* models and investigate the role of LIF in ORN maturation. OMP has served as a marker for ORN maturity because it is expressed exclusively in mature ORNs (31). Moreover, OMP expression is observed only if developing neurons reach the target olfactory bulb (48). The stage that neurons reach the target and form synapses is generally regarded as a critical period for neuronal maturation. Thus, it is reasonable to investigate the maturation of ORNs by using OMP as a marker. We also used another marker, ACIII. ACIII is a critical enzyme in the odorant signal transduction cascade (49), and its expression is restricted in olfactory epithelium to olfactory receptor neuronal sensory cilia (28). Because the development of cilia is another criterion for the maturity of ORNs (39) and ACIII is expressed in the cilia concomitant with ORN maturation (28, 29), ACIII is therefore another appropriate marker of ORN maturity (29, 39). Here, we observed that the expression of OMP and ACIII was increased in LIF-null mice without affecting the total number of neurons in the olfactory epithelium, which indicate that a primary effect of LIF is to "temper" ORN maturity, and thus may support the maintenance of a population of immature neurons.

Here, we propose a novel role for LIF, that it regulates the terminal differentiation of ORNs. Previously, LIF has been implicated in delaying the differentiation of several embryonic cell types, including embryonal carcinoma cells (50) and embryonic stem cells (51, 52). LIF has also been shown to affect neuromuscular junction genesis (53). LIF is expressed in embryonic muscle, and delayed the maturation of the developing motor unit by transiently delaying the withdrawal of excess inputs from polyneuronally innervated myofibrils. LIF may delay the maturation of ORNs in a similar manner, though the direct effect of LIF on olfactory receptor neuronal synaptogenesis is unknown.

LIF mRNA is present throughout the layers of olfactory epithelium in which either mature or immature ORNs exist. Our observation differs from those in a recent immunohistochemical study that concluded that LIF was not expressed in the olfactory epithelium unless injury occurred (21). The discrepancy may reflect a lower sensitivity of detection by immunological methods used in the other study. The specificity of the signal detected by *in situ* hybridization is confirmed by using olfactory epithelium obtained from LIF null mice. The localization of LIF message to neurons of the olfactory epithelium indicates that LIF acts as an autocrine/paracrine factor to regulate differentiation. The components of the receptor that mediates LIF actions and associated downstream signaling molecules are all expressed in the olfactory epithelium as well, supporting an autocrine/paracrine function for LIF. The expression of LIF in mature ORNs may serve as a paracrine factor to deter the terminal differentiation of immature ORNs. Thus, when mature ORNs are lost through normal turnover or damages, the ambient levels of LIF closed to these ORNs are reduced, favoring the maturation of neighboring immature neurons. The expression of LIF in immature ORNs may serve as an autocrine factor to maintain the population of immature ORNs in the olfactory epithelium.

LIF is a member of a family of cytokines that includes IL-6, IL-11, OSM, CNTF, and cardiotrophin 1 (CT-1); these factors are reported to display similar functions in many cases (see review in ref. 8). IL-6 and CNTF showed no significant STAT3 activation in ORN cultures, whereas OSM did activate STAT3. Different from IL-6 and CNTF, OSM binds to two receptor complex forms, OSM receptor/gp130 and LIF receptor β/gp130 with different binding affinities (54). The latter receptor complex is the one activated by LIF, OSM, and CT-1. The role of OSM in the olfactory system *in vivo* has not been reported, so it is unclear that the functional redundancies found between LIF and OSM *in vitro* are representative of their *in vivo* roles (55).

STAT3 mediates the effect of LIF on ORN differentiation. Transfection of cultures with a dominant negative form of STAT3 prevented the ability of LIF to inhibit OMP expression. We hypothesize that activated STAT3 binds directly to the OMP promoter to negatively regulate the expression of genes involved in the terminal differentiation of ORNs. We identified a potential STAT3-binding site in the promoter region of OMP, and demonstrated that active STAT3 indeed binds to this region. Interestingly, the promoter regions of ACIII (−192 to −183) and G_{olf} (−438 to −429) also contain potential STAT-binding sites between the O/E-1 binding sites and transcription initiation sites. These signaling components (ACIII and G_{olf}) are expressed in the cilia of ORNs (28–30) and are central to odorant signal transduction. In contrast, the olfactory cyclic nucleotide-gated channel (OcNC) subunit 1 (56), another important signaling component in odorant transduction (49), does not contain a potential STAT-binding site in its promoter region (−255 to +18). OcNC subunit 1 is expressed much earlier during olfactory neurogenesis than

ACIII, G_{olf}, or OMP (29, 57). The lack of a potential STAT-binding site in the promoter region of OcNC subunit 1 is consistent with our hypothesis that STAT3 mediates expression of proteins relevant to terminal neuronal differentiation.

Our studies support the hypothesis that the ORN population is in a dynamic equilibrium (27). Our working model is that the number of mature ORNs is tightly regulated by feedback mechanisms between neurons at various stages of development. LIF plays an important role in maintaining a population of immature neurons. During neurogenesis, immature neurons not yet in contact with mitral cells in the olfactory bulb acquire sensitivity to LIF; STAT3 activation inhibits the maturation of these cells and may act as a trophic factor for neurons not yet in contact with the olfactory bulb. These neurons are likely required to be available to occupy synaptic spaces vacated by dying mature cells so that olfactory function can be restored quickly (58, 59). As mature neurons senesce and die, trophic factors are released, such as NGF (60), which antagonize the effects of LIF (61, 62) and promote terminal differentiation. The fate of immature ORNs therefore depends on which signal prevails, in this case, either NGF or LIF.

The postnatal neurogenesis that occurs in the olfactory system is not unique, given the discovery that stem cells exist in the adult brain (2, 5, 63). LIF mRNA is highly expressed in dentate gyrus of the adult rat hippocampus (64), where postnatal neurogenesis occurs. Although the role of LIF in this context is unknown, LIF may function in the adult brain to arrest maturation of neuronal precursors. Our results implicate a potential role of neuropoietic cytokines, regulating maturation during postnatal neuronal development.

We thank Dr. S. Landis for LIF null mice and Drs. David Ginty, Hyun Shin, and Stephen Desiderio for critical reading of this manuscript. This work was supported by the National Institute of Neurological Disorders and Stroke (G.V.R.), the National Institute on Deafness and Other Communication Disorders (G.V.R. and C.M.), and the Howard Hughes Medical Institute (J.-Y.Y.).

1. Luskin, M. B. & Shatz, C. J. (1985) *J. Neurosci.* **5,** 1062–1075.
2. Doetsch, F., Caille, I., Lim, D. A., Garcia-Verdugo, J. M. & Alvarez-Buylla, A. (1999) *Cell* **97,** 703–716.
3. Gould, E., Reeves, A. J., Graziano, M. S. & Gross, C. G. (1999) *Science* **286,** 548–552.
4. Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A. & Gage, F. H. (1998) *Nat. Med.* **4,** 1313–1317.
5. Gage, F. H., Ray, J. & Fisher, L. J. (1995) *Annu. Rev. Neurosci.* **18,** 159–192.
6. Huang, E. J. & Reichardt, L. F. (2001) *Annu. Rev. Neurosci.* **24,** 677–736.
7. Murphy, M., Dutton, R., Koblar, S., Cheema, S. & Bartlett, P. (1997) *Prog. Neurobiol.* **52,** 355–378.
8. Taga, T. & Kishimoto, T. (1997) *Annu. Rev. Immunol.* **15,** 797–819.
9. Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kelso, A. & Metcalf, D. (1987) *EMBO J.* **6,** 3995–4002.
10. Murphy, M., Reid, K., Brown, M. A. & Bartlett, P. F. (1993) *Development (Cambridge, U.K.)* **117,** 1173–1182.
11. Murphy, M., Reid, K., Ford, M., Furness, J. B. & Bartlett, P. F. (1994) *Development (Cambridge, U.K.)* **120,** 3519–3528.
12. Bamber, B. A., Masters, B. A., Hoyle, G. W., Brinster, R. L. & Palmiter, R. D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 7839–7843.
13. Ludlam, W. H., Zang, Z., McCarson, K. E., Krause, J. E., Spray, D. C. & Kessler, J. A. (1994) *Dev. Biol.* **164,** 528–539.
14. Rao, M. S., Sun, Y., Escary, J. L., Perreau, J., Tresser, S., Patterson, P. H., Zigmond, R. E., Brulet, P. & Landis, S. C. (1993) *Neuron* **11,** 1175–1185.
15. Mayer, M., Bhakoo, K. & Noble, M. (1994) *Development (Cambridge, U.K.)* **120,** 143–153.
16. Mi, H. & Barres, B. A. (1999) *J. Neurosci.* **19,** 1049–1061.
17. Kurek, J. B., Bennett, T. M., Bower, J. J., Muldoon, C. M. & Austin, L. (1998) *Neuroscience* **249,** 1–4.
18. Cheema, S. S., Richards, L., Murphy, M. & Bartlett, P. F. (1994) *J. Neurosci. Res.* **37,** 213–218.
19. Cheema, S. S., Arumugam, D., Murray, S. S. & Bartlett, P. F. (1998) *NeuroReport* **9,** 363–366.
20. Getchell, T. V., Shah, D. S., Partin, J. V., Subhedar, N. K. & Getchell, M. L. (2002) *J. Neurosci. Res.* **67,** 246–254.
21. Nan, B., Getchell, M. L., Partin, J. V. & Getchell, T. V. (2001) *J. Comp. Neurol.* **435,** 60–77.
2. 2. Mackay-Sim, A. & Chuah, M. I. (2000) *Prog. Neurobiol.* **62,** 527–559.
23. Graziadei, P. P. C. & Monti-Graziadei, M. (1978) in *Neuronal Plasticity*, ed. Cotman, C. W. (Raven, New York), pp. 131–153.
24. Calof, A. L. & Chikaraishi, D. M. (1989) *Neuron* **3,** 115–127.
25. Schwartz Levey, M., Chikaraishi, D. M. & Kauer, J. S. (1991) *J. Neurosci.* **11,** 3556–3564.
26. Caggiano, M., Kauer, J. S. & Hunter, D. D. (1994) *Neuron* **13,** 339–352.
27. Calof, A. L., Hagiwara, N., Holcomb, J. D., Mumm, J. S. & Shou, J. (1996) *J. Neurobiol.* **30,** 67–81.
28. Bakalyar, H. A. & Reed, R. R. (1990) *Science* **250,** 1403–1406.
29. Matsuzaki, O., Bakin, R. E., Cai, X., Menco, B. P. & Ronnett, G. V. (1999) *Neuroscience* **94,** 131–140.
30. Jones, D. T. & Reed, R. R. (1989) *Science* **244,** 790–795.
31. Farbman, A. I. & Margolis, F. L. (1980) *Dev. Biol.* **74,** 205–215.
32. Margolis, F. L. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1221–1224.
33. Ronnett, G. V., Hester, L. D. & Snyder, S. H. (1991) *J. Neurosci.* **11,** 1243–1255.
34. Davis, J. A. & Reed, R. R. (1996) *J. Neurosci.* **16,** 5082–5094.
35. Moon, C., Sung, Y., Reddy, R. & Ronnett, G. V. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 14605–14610.
36. Park, O., Schaefer, T. & Nathans, D. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13704–13708.
37. Escary, J. L., Perreau, J., Dumenil, D., Ezine, S. & Brulet, P. (1993) *Nature (London)* **363,** 361–364.
38. Verhaagen, J., Oestreicher, A. B., Gispen, W. H. & Margolis, F. L. (1989) *J. Neurosci.* **9,** 683–691.
39. Farbman, A. I. (1992) in *Cell Biology of Olfaction*, eds. Barlow, P. W., Bray, D., Green, P. B. & Slack, J. M. W. (Cambridge Univ. Press, Cambridge, U.K.), pp. 167–206.
40. Wang, S. S., Tsai, R. Y. L. & Reed, R. R. (1997) *J. Neurosci.* **17,** 4149–4158.
41. Ihle, J. N. & Kerr, I. M. (1995) *Trends Genet.* **11,** 69–74.
42. Schindler, C. & Darnell, J. E. J. (1995) *Annu. Rev. Biochem.* **64,** 621–651.
43. Darnell, J. E. J. (1997) *Science* **277,** 1630–1635.
44. Levi-Montalcini, R. (1987) *Science* **237,** 1154–1162.
45. Wang, M. M., Tsai, R. Y., Schrader, K. A. & Reed, R. R. (1993) *Mol. Cell. Biol.* **13,** 5805–5813.
46. Behrens, M., Venkatraman, G., Gronostajski, R. M., Reed, R. R. & Margolis, F. L. (2000) *Eur. J. Neurosci.* **12,** 1372–1384.
47. Mackay-Sim, A. & Kittel, P. W. (1990) *Eur. J. Neurosci.* **3,** 209–215.
48. Verhaagen, J., Oestreicher, A. B., Grillo, M., Khew-Goodall, Y., Gispen, W. H. & Margolis, F. L. (1990) *J. Neurosci. Res.* **26,** 31–44.
49. Brunet, L. J., Gold, G. H. & Ngai, J. (1996) *Neuron* **17,** 681–693.
50. Koopman, P. & Cotton, R. G. (1984) *Exp. Cell Res.* **154,** 233–242.
51. Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M. & Rogers, D. (1988) *Nature (London)* **336,** 688–690.
52. Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A. & Gough, N. M. (1988) *Nature (London)* **336,** 684–687.
53. Kwon, Y. W., Abbondanzo, S. J., Stewart, C. L. & Gurney, M. E. (1995) *J. Neurobiol.* **28,** 35–50.
54. Mosley, B., Imus, C. D., Friend, D., Boiani, N., Thoma, B., Park, L. S. & Cosman, D. (1996) *J. Biol. Chem.* **271,** 32635–32643.
55. Jankowsky, J. L. & Patterson, P. H. (1999) *Exp. Neurol.* **159,** 333–346.
56. Dhallan, R. S., Yau, K. W., Schrader, K. A. & Reed, R. A. (1990) *Nature (London)* **347,** 184–187.
57. Margolis, F. L. (1980) in *Proteins of the Nervous System*, eds. Bradshaw, R. A. & Schneider, D. M. (Raven, New York), 2nd Ed., pp. 59–84.
58. Farbman, A. I. (1992) in *Cell Biology of Olfaction*, eds. Barlow, P. W., Bray, D., Green, P. B. & Slack, J. M. W. (Cambridge Univ. Press, Cambridge, U.K.), pp. 24–74.
59. Walker, D. G., Breipohl, W., Simon-Taha, A., Lincoln, D., Lobie, P. E. & Aragon, J. G. (1990) *Chem. Senses* **15,** 741–753.
60. Roskams, A. J. I., Bethel, M. A., Hurt, K. J. & Ronnett, G. V. (1996) *J. Neurosci.* **16,** 1294–1307.
61. Corness, J., Stevens, B., Fields, R. D. & Hokfelt, T. (1998) *NeuroReport* **9,** 1533–1536.
62. Shadiack, A. M., Vaccariello, S. A., Sun, Y. & Zignomd, R. E. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7727–7730.
63. Gould, E., Tanapat, P., Hastings, N. B. & Shors, T. J. (1999) *Trends Cogn. Sci.* **3,** 186–192.
64. Lemke, R., Gadient, R. A., Schliebs, R., Bigl, V. & Patterson, P. H. (1996) *Neurosci. Lett.* **215,** 205–208.